(3)

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68, C07H 21/00, B01J 19/00 — A1

(11) International Publication Number: WO 95/21265

(43) International Publication Date: 10 August 1995 (10.08.95)

(21) International Application Number: PCT/GB95/00209

(22) International Filing Date: 1 February 1995 (01.02.95)

(30) Priority Data:
9401833.0 1 February 1994 (01.02.94) GB

(71) Applicant *(for all designated States except US)*: ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford OX1 3UB (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: SOUTHERN, Edwin, Mellor [GB/GB]; 30 Staverton Road, Oxford OX2 6XJ (GB). MIR, Kalim, Ullah [GB/GB]; 2 Grange Drive, Bradford BD15 7Z (GB). CASE-GREEN, Stephen, Charles [GB/GB]; Flat 2, Sylvia House, 32A Union Street, Oxford OX4 1JP (GB).

(74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, 1 Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: METHODS FOR DISCOVERING LIGANDS

(57) Abstract

A method of identifying one or a combination of ligands, e.g. oligonucleotides or analogues, that interact specifically with a target, e.g. a DNA or an RNA molecule having a secondary or tertiary structure. One ligand may be pre-reacted to open up the target for interaction with other ligands forming an array on a solid surface.

METHODS FOR DISCOVERING LIGANDS

INTRODUCTION

Interactions between molecules form the basis of most biological processes; understanding these interactions is important for the development of applications in basic research and medicine: for example, many drugs act by binding to specific receptor molecules. The task of finding ligands that bind to a given target with high specificity and affinity is often difficult and though the introduction of combinatorial chemistries will make this task easier, it is likely that single ligands for a single biological target may not be effective enough for some purposes; for example, where the aim is to block completely a specific process.

The present invention describes novel ways of discovering combinations of ligands which act together to produce more specific and stronger interaction than can be achieved by a single ligand.

There are two distinct ways in which ligands could act cooperatively:

- Many biologically important macromolecules or macromolecular assemblages, such as proteins and RNA, are held in their active conformation by intramolecular interactions based on weak forces. Binding one ligand to the molecule partially opens its structure, and, as we will show, may expose it to other ligands which cannot bind in the absence of the first ligand. These additional ligands will reinforce the attenuation of the target molecule.

- Many biological processes occur as a result of a series of reactions, each one dependent on

A target is a polymeric molecule having an intramolecular structure such as a nucleic acid (DNA or RNA) or a protein or carbohydrate, or a macromolecular assembly such as a membrane. Targets which are compound molecules such as glycosylated nucleic acids are envisaged. A ligand is a molecule capable of interaction with a target which interaction can form the basis of therapeutic intervention or a biological test. The nature of the interaction is not material to the invention, and may for example be hybridisation or an immune reaction or any other specific binding reaction including covalent binding. Examples of ligands are oligonucleotides, peptides, steroids and glycosides.

The invention is particularly concerned with targets that have secondary or tertiary structure. So far as nucleic acids are concerned, RNA targets often have more structure than DNA targets, and are accordingly preferred. Interaction between a target and ligands on a solid surface is preferably effected under conditions such that the secondary or tertiary structure of the target is retained. For hybridisations between polynucleotide sequences for example, such conditions are well known and are different from the more stringent conditions generally used for standard DNA hybridisation reactions.

Antisense oligonucleotides

Antisense oligonucleotides and RNAs have potential as therapeutic agents, and offer one of very few methods for rational design of therapies for a range of infectious agents including viruses, bacteria and parasites, and for the treatment of cancer. For basic biological studies, they offer a way of finding a link between a gene and its functions. The need for this becomes more important as genome analysis produces

interaction between an oligonucleotide and its target, and so the design of antisense reagents is largely based on informed guesswork. The methods described below provide a rational strategy for choosing antisense reagents based on novel experimental systems which can be used to measure the strength of interaction between a target sequence and all potential antisense reagents. The methods can be used to find those oligonucleotides or analogues which bind most effectively to a target sequence. The methods can be extended to find combinations of oligonucleotides which act cooperatively to open up the structure of the target, enhancing each other's binding. Antisense reagents to different RNAs, providing cooperative attenuation of the activity in a biological process involving more than one gene product, can readily be found by analysing the RNAs in the way described. The same methods can be used to find antisense targets in cells where nothing is known about specific gene expression. These targets may be single RNAs or mixtures.

The methods to be described provide an entirely new approach to antisense design. It can readily be seen that the methods described for systems based on nucleic acids could be adapted to any other system for which suitable chemical procedures already exist, or could be developed.

Arrays of ligands

To test interactions between a target molecule and large number of ligands, it is convenient to make the ligands on the surface of a solid substrate, where they can all be reacted and analysed simultaneously. Large arrays of synthetic oligonucleotides can be made on the surface of a glass plate or plastic sheet [Maskos and Southern, 1992a,

sequence at each step. A single procedure, equivalent to synthesising one long oligonucleotide, creates a set of oligonucleotides which represent the entire sequence of the target molecule; each oligonucleotide in the set represents a "window" on the sequence. The width of this window can be predefined - typically we use a window of 10-15 bases and it is an important feature of the method for making scanning arrays that oligonucleotides of all lengths from a single base to this chosen length are made in the same process (Fig. 2).

Effects of intramolecular structure on ligand binding

Many biological macromolecules, including proteins, RNA and DNA, are folded into relatively stable structures which are important for biological activity. The structure is dependent on the primary sequence of the macromolecule, and also on weak intramolecular interactions based on hydrogen bonding, hydrophobic interactions etc. The macromolecule may also be bound up with other molecules, as for example would be a mRNA with proteins in a cell. A consequence of molecular folding is that some residues in the molecule are more accessible to interaction with ligands than others; this is of great importance in the design of drugs, which must bind to their target molecule.

We illustrate the problem of finding ligands that can bind to a folded macromolecule with reference to $tRNA^{phe}$, a molecule with well characterised structure. First, labelled RNA was hybridised to a general array of the type $N_3X_2N_3$, under non-stringent conditions which would retain the folded structure of the RNA molecule. The pattern revealed that only a few regions of the 76 base sequence were open to hybridisation (Fig. 3 and the legend which describes the construction of the array).

of antisense reagents, some apparently open regions do not interact with complementary oligonucleotides.

The strength of interaction, measured from the intensity of hybridisation, is very variable, with more than 10000-fold ratio of the strongest to the weakest. It is a major benefit of the technique that the strength of interaction can be seen immediately from examination of the hybridisation pattern. It is also possible to distinguish effects due to structure in the target from those which are due to structure in the oligonucleotides; this is important information for making choices of oligonucleotides for antisense applications. A striking feature of all analyses we have done with natural RNAs is that only a very small number of sites interact strongly with complementary oligonucleotides, and we often find that the strength of interaction changes abruptly as a result of adding or subtracting a single base (e.g. the HIV-TAR analysis shown in Fig.2b). These patterns of hybridisation, which are crucially important for the design of antisense reagents, could only be predicted from theory if a great deal were known about the structure of the RNA molecule, and if the algorithms used to calculate the relative strength of interaction of different oligonucleotides were greatly improved over those available at present. There is no other experimental system that could give the information provided by the arrays.

Identification of ligands which act cooperatively on a folded target

Studies of a number of mRNAs using the method described above show that most bases are unavailable for pairing with oligonucleotides. However, when a first oligonucleotide does bind it is likely to break internal structures, releasing other bases from

Antisense reagents are not normally composed of oligonucleotides made from natural nucleotides, but from analogues. The important features are that:

1. They should bind specifically to a target sequence.
2. They should be able to enter the target cell.
3. They should be resistant to degradation in the cell.
4. They should induce either breakdown of the target nucleic acid or block its function.

These criteria are matched better by some modified oligonucleotides and/or analogues than by natural oligonucleotides. The chemistry that is used to synthesise analogues is readily adaptable to making arrays, and so the strategy described here can be adapted to any analogue. Furthermore, there are numerous potentially desirable modifications, for example the addition of moieties which would favour penetration of the cell or enhance binding to the target molecule whose effects on binding are not readily predictable. The array methods would allow these measurements to be carried out in a simple and straightforward way, provided that a synthetic route could be found to make the necessary structure on the array; this is the case for most of the useful modifications known at the present time.

Other ligand/target interactions

Clearly, the principles illustrated by reference to antisense oligonucleotides could be used to discover combinations of ligands of other types. The essentials are:

- It must be possible to make an array of one of the potential ligand types in which the identities of the ligands occupying different cells of the array are known.

solution, this would be $4^{16} = 4 \times 10^9$.

Linked ligands

Pairs or higher multiples of ligands act to increase binding to a folded molecule by inhibiting the competing reaction - reformation of the internal structure of the target molecule. They will also increase the rate of binding by opening the structure of the target. Both of these processes could be enhanced if the ligands were joined so that trimolecular or higher order interactions were reduced to bimolecular interactions. It is easy to imagine that a pair of oligonucleotides, including short ones such as di-, tri- and tetra-nucleotides, or other ligands, could be joined by a flexible linker that would allow them both to bind. The effect of linking the interacting moieties together could be further extended by enhancing the binding of one or more of the ligands to the target, for example, by covalent cross-linking, by chelation, by intercalation, or by charge or van der Waals interaction with the target molecule. This would allow other ligands more time to bind to the target. Linkers with other desirable qualities, such as greater cell permeability, could further improve the ligands' properties. A ligand could form the basis of a ribozyme, e.g. by having at least one portion for interacting with a target and another portion with catalytic activity.

EXAMPLES

Apparatus for making arrays

The device used to form the cell must make a good seal against the substrate on which synthesis takes place. We use glass for the substrate. Teflon is the only material we have found which makes a good

Oligonucleotide synthesis used standard reagents for phosphoramidite chemistry, omitting the capping step. The ABI 381A was programmed to couple bases in the order corresponding to the complement of the target sequence, with an interrupt after deprotection. The scale was for 0.2 μmol. synthesis, adjusted slightly to provide volumes that would just fill the reaction chamber.

Final deprotection in 30% ammonia was carried out in a specially constructed bomb, comprising a chamber (230 x 230 x 20mm) cut into a Nylon block (300 x 300 x 30mm), sealed by a sheet of silicone rubber (3mm thick), compressed against the rim of the chamber by clamping the whole assembly between two mild steel plates (6mm thick) using four bolts along each side of the square. After 5-8h at 55°C the bomb was cooled to 4°C before opening. The plate was then washed in ethanol followed by Tris/EDTA (0.01M, pH 7.8, 0.1% SDS) and ethanol and then dried in an air stream.

Hybridisation reactions

We have used a variety of target molecules in experiments with scanning arrays: synthetic oligonucleotides labelled using polynucleotide kinase with gamma-^{32}P, gamma-^{33}P or gamma-^{35}S-ATP to tag the 5' end; RNAs labelled at the 3' end using RNA ligase with 5'-^{32}P cytosine-3',5'-diphosphate; or transcripts of DNA fragments made from PCR amplified fragments using T7 or SP6 polymerase to incorporate α-^{32}P or α-^{35}S UTP. All of these make good hybridisation targets. Most hybridisation reactions were carried out at 4-25°C, in solutions containing 3-4.5M TMACl or 1.0 M NaCl. After hybridisation, the plate was rinsed in the hybridisation solvent and exposed through Clingfilm to a storage phosphor screen (Fuji STIII) which was then scanned in a Molecular Dynamics 400A

increasingly wide rows and columns such that each successive row or column embraces four from the previous layer. In all cases, the array comprises all 4^s possible oligonucleotide sequences of length s. The results of hybridisation to such an array are shown in Fig. 3. More complex arrays can be made by applying mixtures of all four bases at certain stages in the synthesis; for example, all octanucleotides can be made in 4096 cells by applying four mixed bases in the two central positions, to produce an array of the type $N_3X_2N_3$. In this case, each cell comprises a mixture of 16 different octanucleotides. The results of hybridisation to such an array are shown in Fig. 3.

Figure 2a.

Scanning arrays are made by applying oligonucleotide precursors to the glass substrate in a circular patch. The first base, corresponding to the complement of the 5' end base of the target sequence, is applied to the left end of the plate, which is then moved by a predetermined off-set, and the second base added. The process is repeated for the whole of the sequence. The length of oligonucleotides made on the centre line is equal to the diameter of the cell divided by the offset, in this case, diam. = 30mm and offset = 2.5mm, giving a maximum overlap of 30/2.5 = 12. Oligonucleotides ranging in size down to a single base are made in the segments flanking the centre line.

The upper panel shows a scanning array representing the 76 bases of $tRNA^{phe}$, hybridised to end labelled tRNA. Note the strong hybridisation in the region of the D-loop, more moderate hybridisation to the variable loop, and very weak hybridisation to the anticodon loop and the 5'-end of the molecule.

Much is known about the rabbit globin system. It was one of the first mRNAs to be isolated and characterised by translation in vitro. We have studied it because we wish to use it to study it as a model for antisense intervention. In preparation, we made a scanning array of oligonucleotides complementary to 122 bases around the initiation codon of the mRNA (atg marked in bold letters at bases 54-56). The folded structure shown in the figure is that with minimum free energy calculated by the energy minimisation program mfold, and displayed by squiggles. Hybridisation of labelled RNA showed interaction at the region complementary to bases 46-62 marked by a thick line on the molecule. We then made a deoxyribooligonucleotide corresponding to this 17-mer. Rehybridisation of the complex showed, as expected, no signal at the position of bases 46-62, but new signal at bases 32-45, marked with a dashed line. This is a surprising result, as there is no indication from the computer prediction that bases 46-62 interact with bases 32-45. On the contrary, there is a single stranded region of nine bases between them, which should be enough to decouple any interaction. Thus, the experiment suggests an interaction relevant to the design of antisense reagents which could not have been predicted by energy calculations or by examination of the computer generated structure.

Figure 6.

Four arrays were made of complements to the same sequence, a region of the human CFTR gene (mutations in this gene are responsible for cystic fibrosis). The arrays were made as described in Southern et al (1994) on a glass substrate derivatised with a hexaethylene glycol linker.

a) natural deoxyribonucleotides tethered to the

that is arrays comprising all oligonucleotides of a chosen length. In this experiment, we used two types of universal arrays, one comprising all 4096 hexanucleotides (Fig. 7b), the other all 65,536 octanucleotides (Fig. 7c). In the latter array there were only 4096 cells, each containing a mixture of sixteen different octanucleotides of the general form NNNXXNNN, where N is a defined base, and X is a mixture of all four. These universal arrays do not indicate the optimum oligonucleotide to use, as they only have sequences of limited length, but they do reveal the regions in the target sequence which are most available for hybrid formation. These areas were then analysed in more detail using scanning arrays as described below. The method used to make the universal arrays produces oligonucleotides half the length of the major oligonucleotides in lines which intersperse them. A surprising result of the analysis is that some of these tri- and tetranucleotides interact strongly with the target (see the lines of uniform intensity in Figs. 7a and b). With other targets we see the same feature, but with different oligonucleotide sequences. In one case (Southern et al, 1994), we have seen interaction between a target and a dinucleotide. It is likely that these interactions are the result of the folding of the target RNA, which may present short stretches of sequence in a structure which is particularly favourable for interaction with oligonucleotides; for example, a stacked half helix. These observations suggest a novel approach to antisense design, based on the use of array analysis. Namely, that short oligonucleotides, which are seen to interact in this structure specific manner can be incorporated in the cocktail of antisense agents specific to the target, either as a component of a mixture, or as a component of a linked composite molecule.

for HIV. We have analysed its hybridisation behaviour with arrays of natural oligodeoxyribonucleotides, and with an array made with analogues that are commonly used as antisense agents, 2'-O-methylribonucleotides (Fig. 11c). The same region is found to hybridise in both cases, the loop of the stem-loop, but only one or two sequences in this region form duplex, and the exact position of the sequence with strongest interaction is different with each analogue. In an experiment with the natural oligodeoxyribonucleotides, we selected an antisense sequence as the "pioneer" (Fig. 2b and 11a) and used it to open the structure. Note the loss of binding in the region corresponding to the solution ligand, and the additional binding at other sites. When the complex was bound to the array, several oligonucleotides which had not bound in its absence, now gave a significant yield of duplex (Fig. 11b).

Conclusion

The above examples illustrate several new approaches to the design, characterisation and discovery of ligands made possible by analysis on arrays on solid supports. We have shown how this approach can:

- Identify regions of the target that are open to interaction with ligands;
- Identify combinations of ligands that act together to give a different, usually stronger, interaction than either alone;
- Discover regions of the target that are structured in such a way as to allow them to bind in unexpected ways, e.g. target RNAs to very short oligonucleotides;

The arrays and the cooperative ligands used in the examples were made of a variety of chemical types, illustrating the generality of the approach.

8. Southern, E.M., Maskos, U. and Elder, J.K. (1992). Analysis of Nucleic Acid Sequences by Hybridization to Arrays of Oligonucleotides: Evaluation using Experimental Models. Genomics 12: 1008-1017.

9. Southern, E. M., Case-Green, S. C., Elder, J. K., Johnson, M., Mir, K. U., Wang, L. and Williams, J. C. Arrays of complementary oligonucleotides for analysing the hybridisation behaviour of nucleic acids. Nucleic Acids Research 1994, Vol. 22, No. 8, 1368-1373.

10. Matson, R. S., Rampal, J. B. and Coassin, P. J., Biopolymer synthesis on polypropylene supports I oligonucleotides, Analyt. Bioch. 217, 306-310 (1994).

7. A method as claimed in any one of claims 4 to 6, wherein the ligands are oligonucleotide analogues modified by the addition or substitution of other chemical moieties selected from oligoaliphatic ethers, intercalating agents, positively charged residues, chelating agents and lipophilic agents.

8. A method as claimed in any one of claims 1 to 7, wherein the ligands form the basis of a ribozyme.

9. A method as claimed in any one of claims 1 to 8, wherein the target and one or more ligands are different chemical types.

10. A method as claimed in any one of claims 1 to 9, wherein at least one ligand becomes covalently bound to the target.

11. A method as claimed in any one of claims 2 to 10, wherein the at least one ligand to be bound to the target to form a target complex in step a), is chosen by mixing the target with a library of ligands and choosing from the library at least one ligand that binds to the target.

12. A method as claimed in any one of claims 1 to 11, wherein the target is an RNA.

13. A method as claimed in any one of claims 1 to 12, wherein the target is a molecule having a secondary or tertiary structure, and is caused to interact with the array of ligands under conditions such that the secondary or tertiary structure is retained.

Fig.2a.

Analysis on scanning arrays

tRNA^{phe}

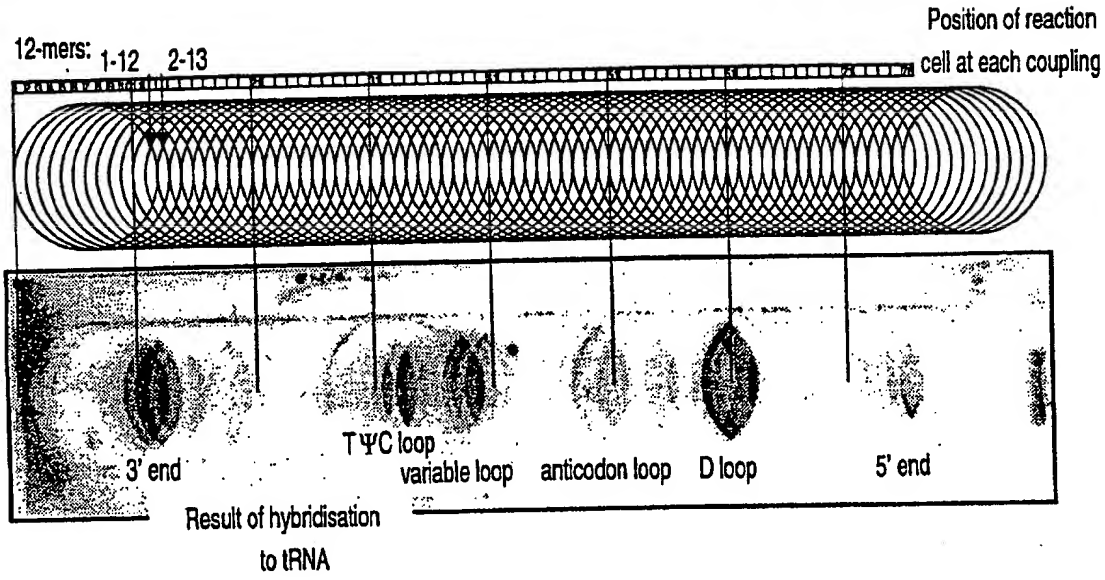


Fig.2b.

HIV TAR

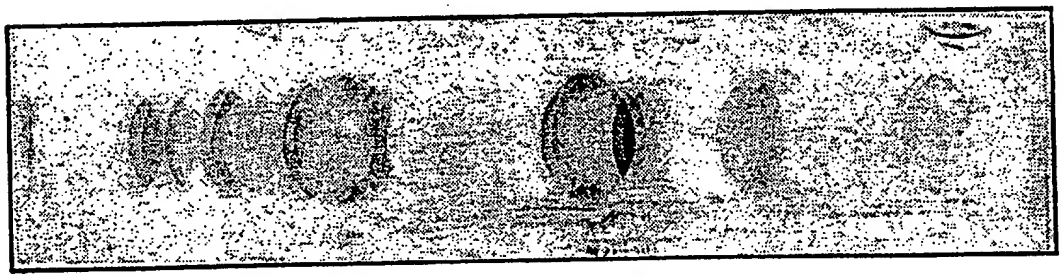


Fig.4.

tRNA with cooperative antisense interactions

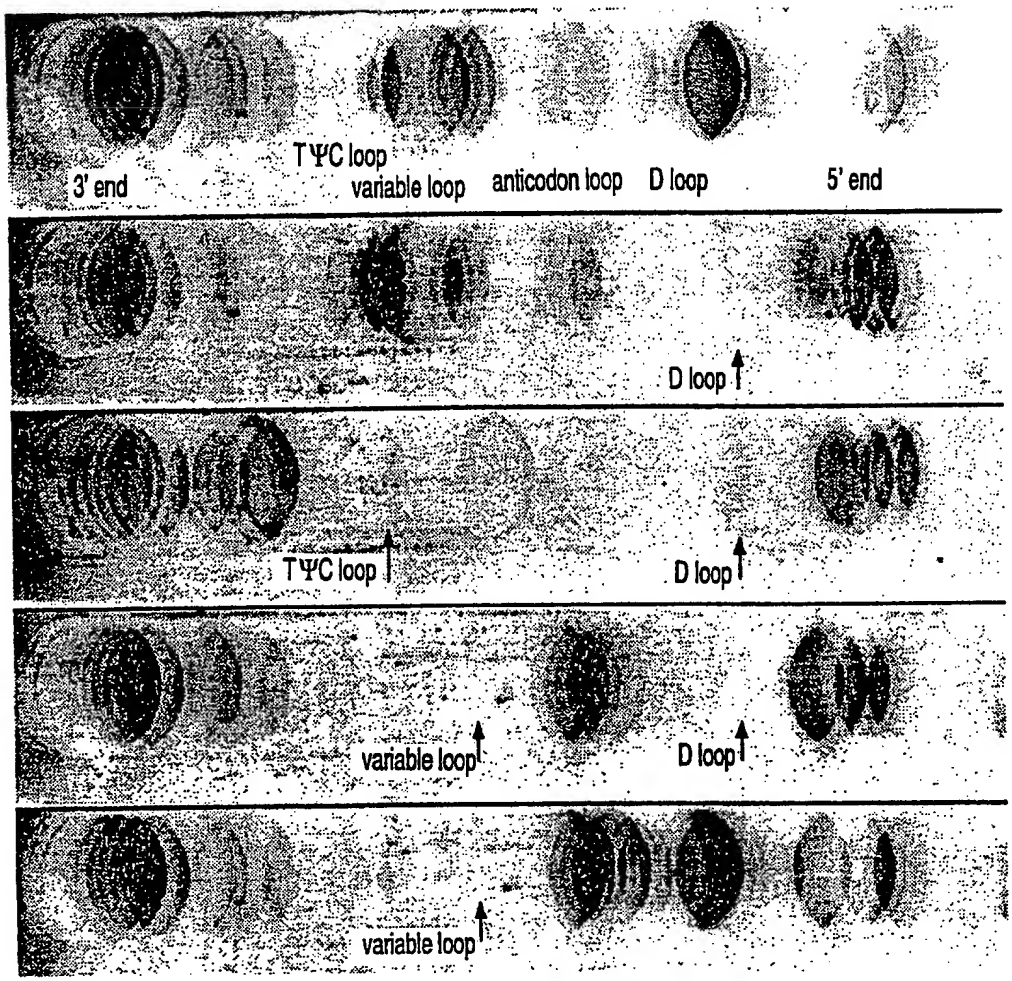


Fig.6.

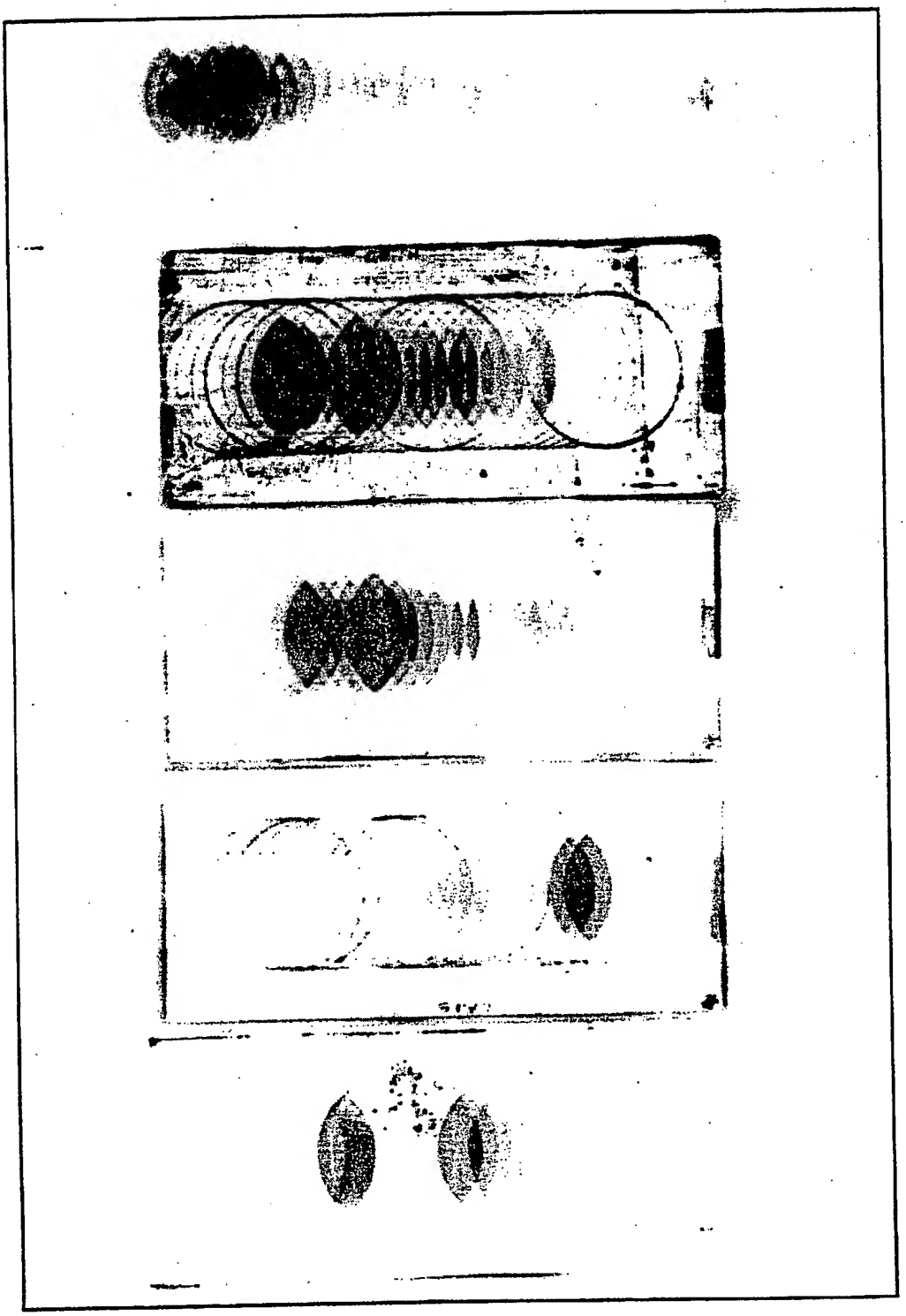


Fig.7b.

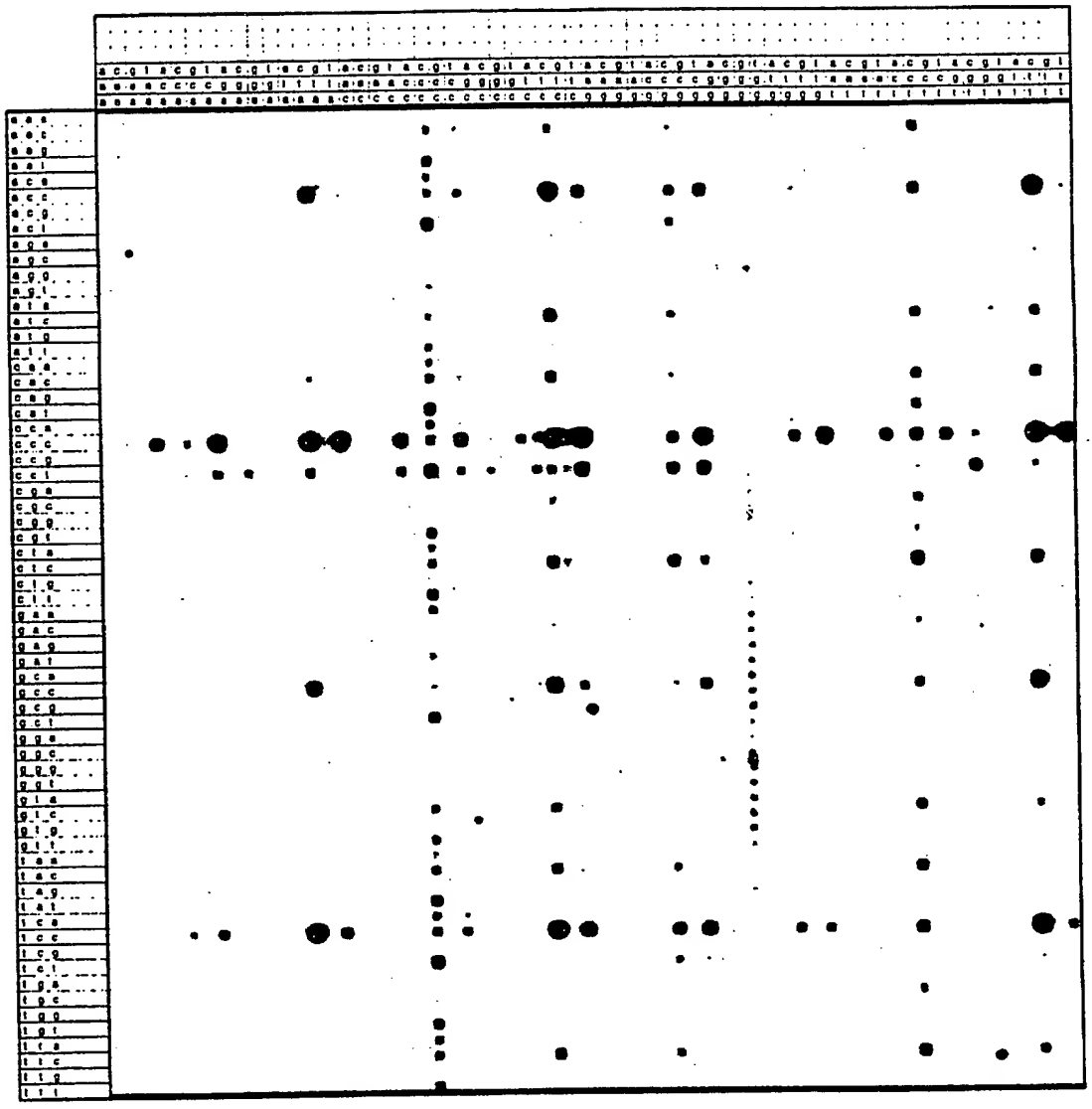


Fig.8a.

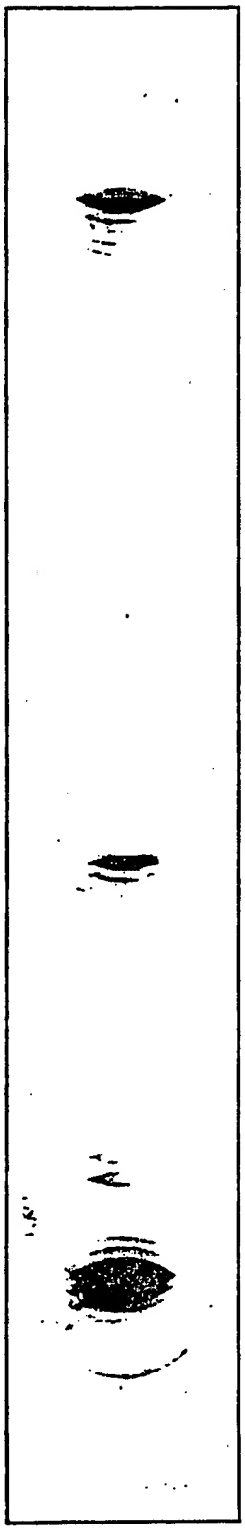


Fig.8b.

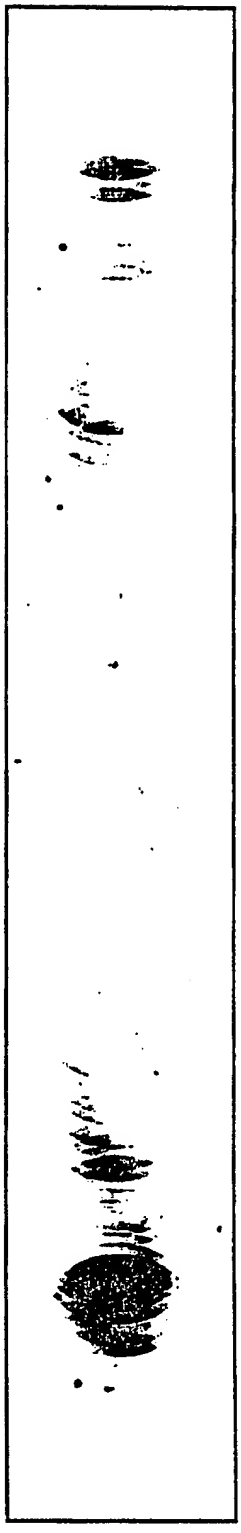


Fig.9a.

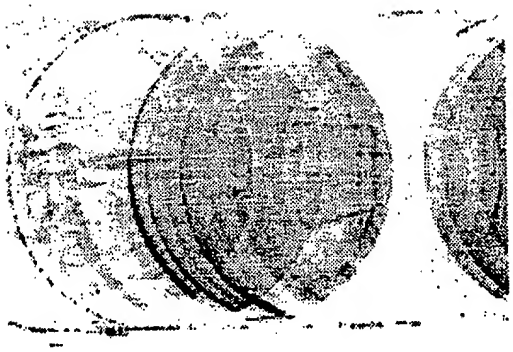


Fig.9b.

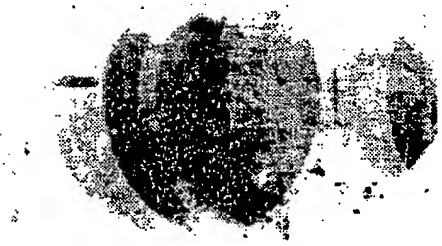
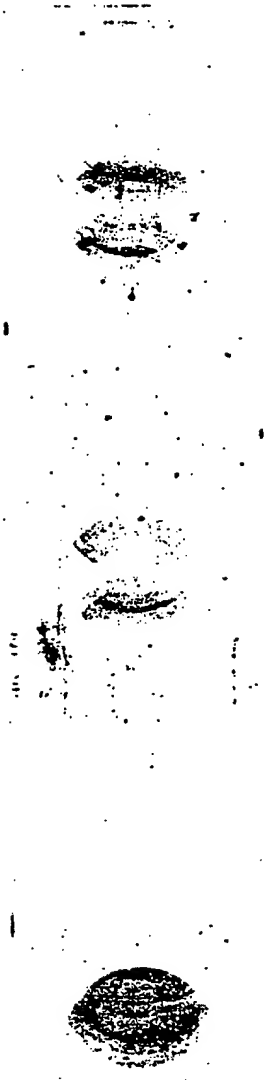


Fig.11a.

Fig.11b.



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/00209

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C07H21/00 B01J19/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 B01J C12Q C07K C07H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,93 22678 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY ET AL.) 11 November 1993 see page 1, paragraph 1 see page 3, line 21 - page 5, line 12 see page 7, line 27 - page 9, line 18 see page 13, line 11 - page 14, line 20 see page 24, line 6 - line 21 see page 35, line 19 - page 36, line 30 see page 40, line 5 - line 15 see table 3 ---	1,4,7,12
P,X	WO,A,94 05394 (ARRIS PHARMACEUTICAL CORPORATION) 17 March 1994 see abstract see page 1, paragraph 1 see page 6, line 19 - page 7, line 39 see page 12, line 39 - page 15, line 26 --- -/--	1,4,9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 May 1995	31. 05. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Stevnsborg, N

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter[illegible]al Application No
PCT/GB 95/00209

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9322678	11-11-93	EP-A-	0638173	15-02-95
WO-A-9405394	17-03-94	AU-B-	4844593	29-03-94
		AU-B-	6393994	14-09-94
		WO-A-	9419694	01-09-94
US-A-4921788	01-05-90	NONE		
WO-A-9322680	11-11-93	AU-A-	3148193	15-06-93
		AU-B-	4110793	29-11-93
		CA-A-	2124087	27-05-93
		EP-A-	0624059	17-11-94
		WO-A-	9309668	27-05-93
US-A-5270163	14-12-93	AU-A-	8206191	07-01-92
		EP-A-	0533838	31-03-93
		WO-A-	9119813	26-12-91